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Analysis of human interleukin-5 gene transcription by a novel nuclear run on method based on the polymerase chain reaction

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Abstract

The total abundance of any mRNA is determined by several factors, principally the rate of new gene transcription and the stability of the mRNA. Interleukin-5 (IL-5) is a cytokine with an important role in supporting the proliferation, survival and activation of eosinophils. Gene transcription of IL-5 mRNA in human T cells was assessed by the conventional nuclear run on assay, but the signal strength was too low for satisfactory analysis. A novel run on assay was developed in which nuclei were incubated with and without nucleotides, and transcripts were detected by reverse transcription-polymerase chain reaction (RT-PCR). The difference between the samples with and without nucleotides was a measure of the amount of new transcription. IL-5 gene transcription was not detected in unstimulated T cell line HSB-2 cells or in unstimulated human T cells prepared from peripheral blood. Transcription was rapidly induced by a variety of stimuli, and ceased by 4–6 h after activation. This method is applicable to other genes expressed at low abundance, such as cytokine genes. mRNA stability was measured by quantitative RT-PCR. After activation with phorbol myristate acetate and ionomycin, the half-life of IL-5 mRNA was 2.6 h in HSB-2 cells and 4.0 h in T cells prepared from human blood. These data, taken together, indicate that human IL-5 mRNA is predominantly regulated at the level of gene transcription.

Keywords: Cytokine; Gene expression; T lymphocyte; RNA processing

1. Introduction

The ultimate steady-state level of any mRNA species is dependent on the interplay of various elements influencing the rate of gene transcription, the processing of transcripts and the rate of mRNA degradation. The nuclear run on (run off) assay is currently the most sensitive assay for measuring specific gene transcription and allows the direct measurement and comparison of gene transcription in various cells in response to different stimuli and/or inhibitors (McKnight and Palmiter, 1979; Greenberg and Ziff, 1984). This assay is often used to assess whether changes in the mRNA levels of a particular gene reflect a change in its synthesis as opposed to a change in the rate of mRNA degradation, mRNA processing or nucleocytoplasmic transport of mRNA. The procedure involves the isolation of cell nuclei and incorporation f nucleotides labelled with ^{32}P into nascent RNA transcripts. The transcripts are

Abbreviations: PBMC, peripheral blood mononuclear cells; RT-PCR, reverse transcription-polymerase chain reaction

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then detected by dot blot hybridisation. However, the assay requires the preparation of large numbers of nuclei and it is not sufficiently sensitive to study transcription of genes expressed at low abundance.

Interleukin-5 (IL-5) is a cytokine which in humans selectively induces the proliferation and differentiation of eosinophils (Clutterbuck et al., 1989; Ema et al., 1990). IL-5 mRNA was detected in the bronchial mucosa of asthmatic patients with eosinophilia but not in those patients with no eosinophil infiltration nor in normal controls (Hamid et al., 1991). Recent work using double immunocytochemistry and in situ hybridisation, revealed that the primary source of IL-5 in bronchial biopsies and bronchoalveolar lavage cells in atopic asthmatics was activated T cells, representing greater than 70% of the positively staining cells (Ying et al., 1995). In animal models of pulmonary hyperresponsiveness, injections of monoclonal antibodies to IL-5 completely suppressed the blood and tissue eosinophilia and bronchial hyperresponsiveness in response to allergen challenge (Okudaira et al., 1991). Likewise in mice with disrupted IL-5 genes, eosinophilia and bronchial hyperresponsiveness could not be induced (Foster et al., 1996). Despite its biological importance, human IL-5 is expressed in low abundance. Its protein is only produced by a few percent of T cells from peripheral blood after activation (Jung et al., 1995). mRNA for IL-5 was not detected by Northern blotting of activated human PBMC under conditions that readily detected mRNA of the related cytokines IL-3 and GM-CSF (Guiffre et al., 1993).

Previous studies on human IL-5 mRNA expression have demonstrated induction of IL-5 mRNA accumulation after activation of human T lymphocytes via the T cell receptor. Protein synthesis is also required (Staynov and Lee, 1992; Van Straaten et al., 1994). However, no previous studies have investigated whether these increases in mRNA expression in human T lymphocytes are a result of increased rates of gene transcription or enhanced IL-5 mRNA stability. We have investigated the effects of various stimuli on the rate of IL-5 gene transcription and on total IL-5 cellular mRNA expression in human T lymphocytes. Conventional nuclear run on assays were performed, but were unable to detect IL-5 gene transcription. Therefore the PCR was employed to develop a novel nuclear run on assay, which readily detected inducible transcription of the human IL-5 gene in response to a variety of stimuli.

2. Methods

2.1. Cell culture

The human T leukaemia cell line HSB-2 was maintained in complete medium consisting of RPMI 1640 (Gibco-BRL) supplemented with 10% (v/v) heat inactivated foetal calf serum (CSL, Melbourne Australia), 2 mM glutamine (Gibco-BRL), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL) and 20 mM HEPES (CSL). Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Aliquots of PBMC (1×10^6 cells/ml) were stimulated with phytohaemagglutinin (PHA, Burroughs-Wellcome, Dartford UK) (1 μg/ml) on day 0, diluted 5× with complete medium supplemented with human rIL-2 (5 U/ml) (Boehringer Mannheim, Germany) on day 3, and maintained in culture for an additional 4 days. These cells will be referred to as 7-day-cultured PBMC, and represent a population of 99% T cells as demonstrated by cell surface phenotyping.

2.2. Isolation of nuclei

HSB-2 cells or 7-day-cultured PBMC were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) (4 ng/ml) and/or ionomycin (Calbiochem, La Jolla, CA, USA) (1 μg/ml). 7-day-cultured PBMC were also stimulated with PHA (1 μg/ml) or with rIL-2 (10 U/ml) for 3 h. Cells were harvested at 400 × *g* for 5 min at 4°C, washed 3 times with ice-cold PBS and resuspended in 5 ml of ice-cold lysis buffer (3 mM $MgCl_2$, 1 mM KCl, 10 mM Tris-HCl, pH 7.4) containing 0.3% NP-40 (Sigma). Cells were incubated at room temperature until lysed and then layered on 10 ml of sucrose cushion (30% sucrose in lysis buffer containing no NP-40). Following centrifugation at 600 × *g* for 10 min at 4°C, the nuclear pellet was resuspended in 100 μl of nuclear storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5

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mM $MgCl_2$, 0.1 mM EDTA). Nuclei were either stored in liquid nitrogen, or used immediately.

2.3. Nuclear run on transcription assay

Nuclear run on assays were performed as described (Naora and Young, 1994). Fresh or thawed nuclei (100 μl) were incubated for 30 min at 30°C in a total volume of 200 μl containing 20% glycerol, 30 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, 0.5 mM each of rATP, rCTP and rGTP, 100 μCi [α-^{32}P]rUTP (3000 Ci/mmol, Amersham) and 40 U RNasin (Promega, Madison, WI). Any contaminating DNA and protein was removed by successive incubations with 40 U of RQ1 RNase free DNase (Promega) at 37°C for 30 min, followed by proteinase K (200 μg/ml) in 1% SDS, 5 mM EDTA and 10 mM Tris-HCl, pH 7.4, at 37°C for 45 min. The samples were then phenol/chloroform/isoamylalcohol (25/24/1) extracted and ethanol precipitated twice to remove any unincorporated radionucleotides. The RNA was resuspended in 100 μl of TES solution pH 7.4 (10 mM (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid) pH 7.4; 10 mM EDTA; 0.2% SDS).

Plasmids containing cDNA for human IL-5 and GAPDH cDNA were provided by Dr I. Young (John Curtin School of Medical Research, Canberra) and Dr G. Goodall (The Hanson Centre for Cancer Research, Adelaide), respectively. 5 μg of the IL-5 308 bp *StyI/BglII* fragment or the GAPDH 153 bp *SacII/XbaI* fragment were incubated with an equal volume of 1 M NaOH at 37°C for 10 min. The DNA solutions were then applied to Hybond-N + nylon membrane (Amersham, Amersham, UK) and dried. The plasmids were hybridised to the nuclear run on transcripts as described (Naora and Young, 1994) with a few modifications. Filters were prehybridised overnight at 65°C in TES: NaCl solution (TES solution, 1 × Denhardt's solution and 200 μg/ml denatured yeast transfer RNA). Fresh hybridisation solution was added prior to the addition of nuclear run on transcripts and hybridisation performed for 36 h at 65°C. The filters were then washed in 2 × SSC at 65°C for 1 h, incubated at 37°C in 2 × SSC containing 20 μg/ml of RNase A (Sigma) for 30 min, and washed in 2 × SSC at 37°C for 1 h. Transcription was analysed by autoradiography at −70°C.

2.4. PCR based nuclear run on assay

Fresh or thawed nuclei (200 μl) were split into two aliquots and incubated for 30 min at 30°C in 20% glycerol, 30 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 1 mM DTT and 40 U of RNasin (Promega). 0.5 mM each of rATP, rCTP rGTP and rUTP were added to one aliquot. No rNTPs were added to the second aliquot. After 30 min, the nuclei were lysed by the addition of 200 μl of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. 20 μg of yeast tRNA was added and RNA was extracted by the acid-guanidinium-thiocyanate method as described (Chomczynski and Sacchi, 1987) and resuspended in water treated with diethylpyrocarbonate (DEPC).

cDNA was synthesized as described (Rolfe et al., 1992). In brief, RNA was heated to 65°C for 5 min and made up in 50 μl with dNTPs at 250 μM each, 200 ng of oligo$(dT)_{12-18}$ (Pharmacia), 4 U AMV Reverse Transcriptase (Pharmacia) and 1 × RT buffer (Pharmacia). The samples were incubated at 42°C for 60 min then heated to 65°C for 5 min. PCR amplification and detection of products were performed as previously described (Rolfe et al., 1992). In brief, reaction mixtures contained 200 μM of each dNTP, 250 ng of each primer, 1 U of *Taq* DNA Polymerase (Boehringer Mannheim, Mannheim, Germany) in 1 × PCR buffer (Boehringer Mannheim) to a volume of 50 μl. IL-5 was amplified for 35 cycles and β-actin for 30 cycles of 95°C for 1 min, 60°C for IL-5 or 58°C for β-actin for 30 s, and 72°C for 30 s, using a Gene Machine (Innovonics, Victoria). Comparisons were made with different amounts of starting material to ensure that PCR products were still accumulating exponentially when the reactions were terminated. Oligonucleotide primers were designed to bind to exons and to span one or more introns, so that cDNA from heteronuclear RNA would yield a larger PCR product than cDNA from fully spliced RNA. The hybridisation primers were designed to bind to a region of cDNA between the two amplification primers. The primer sequences were, IL-5, amplification primers 5'CGGATCCCACAGAAATTCCCACAA3' and 5'TGATATCCACTCGGTGTTCATTAC3'; hybridisation primer 5'CTTCAGTGCACAGTTGGTGAT3'; β-actin, amplification

primers 5'TCACCAACTGGGACGACATG3' and 5'GTACAGGGATAGCACAGCCT3'; hybridisation primer 5'CAGCCATGTACGTTGCTATC3'. In some experiments, total cellular RNA was extracted from an aliquot of cells at the time the transcription reactions were set up. RNA was extracted, reverse transcription was performed and 4×10^5 cell equivalents of cDNA were subjected to RT-PCR.

The PCR products were size fractionated by electrophoresis in 1.2% agarose (SeaKem ME, FMC Bioproducts, Rockland MA, USA). PCR products were detected either by staining with ethidium bromide or by Southern hybridisation after alkaline transfer to Hybond-N+ nylon membrane, and autoradiography at −70°C using Kodak X-ray film (Eastman Kodak, Rochester, NY). Blots were prehybridised in 7% SDS, 0.25 M NaP_i, pH 7.2, 1 mM EDTA for 2 h at 50°C. 120 ng of hybridisation primer, end labelled with ^{32}P using T4 polynucleotide kinase (Pharmacia), was added overnight at 50°C. Blots were washed twice with 2 × SSC 0.1% SDS at room temperature for 5 min, and once with 1 × SSC 0.1% SDS at 50°C for 15 min. The intensities of the bands from the autoradiographs or negatives were quantitated by scanning densitometry (LKB densitometer, Sweden) or using the NIH *image* program (version 1.55; Wayne Rasband, NIH, USA).

2.5. mRNA stability studies

Plasmid pSPAG2 (kindly provided by A. Guiffre, Haematology Dept. St. Vincents Hospital), was derived from pSP64(polyA) and contains the binding sequences for the two human IL-5 amplification primers described above (Guiffre et al., 1993). Competitor RNA (cRNA) was generated by linearising the plasmid with *Eco*RI and performing a transcription reaction using an SP6 Riboprobe Transcription System (Promega). The resultant cRNA was separated from the template DNA by band interception after electrophoresis through a 4% followed by a 3% Nusieve agarose gel (FMC Bioproducts). Bands were detected by UV light and excised from the gel, melted at 67°C for 10 min, diluted in DEPC-treated water, cleaned by repeated phenol extractions, and quantitated by absorbance at 260 nm.

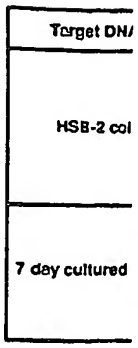
cDNA synthesis was carried out in a final volume of 48 μl and contained 10 μl of RNA consisting of 5 μl of total cellular RNA (2×10^5 cell equivalents) and an equal volume of known numbers of cRNA molecules. The other components of the RT reaction are described above. cDNA was then subjected to PCR for 35 cycles, using the conditions described above. Amplification of IL-5 cRNA and IL-5 mRNA yields PCR products of 103 bp and 328 bp, respectively. PCR products were separated by agarose gel electrophoresis, transferred to Hybond-N+ nylon membrane, and hybridised using two labelled primers, the wild-type IL-5 hybridisation primer (described above) and the cRNA hybridisation primer (5'GATGGCAGCAACGGAAAGTT3') (Guiffre et al., 1993). Hybridisation conditions were as described above.

Laser densitometry was used to calculate the signal strength of both IL-5 mRNA and IL-5 cRNA bands. The ratios of IL-5 cRNA to IL-5 mRNA were calculated for each point. A graph plotting the number of cRNA molecules against the cRNA/mRNA ratio was generated and the line of best fit was generated by linear regression analysis. The number of cRNA molecules where the ratio equals 1 was calculated, and this was determined to be the number of IL-5 mRNA molecules. To derive RNA stability, the number of IL-5 RNA molecules was plotted against the time in hours and mRNA half-life was calculated using linear regression analysis. Values were expressed as the mean and standard error of triplicate experiments. All statistical analyses were performed using Student's paired *t*-test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Conventional nuclear run on assay

HSB-2 cells were activated with PMA and ionomycin, which induces a marked increase in total cellular IL-5 mRNA within 3–4 h (J.E. Valentine and W.A. Sewell, unpublished observations). 4 h after activation, nuclei were extracted and subjected to the conventional nuclear run on assay for 30 min. Nuclei were lysed and RNA was extracted and hybridised to membrane-bound fragments of IL-5 cDNA. No IL-5 transcription was detected, even when very high cell numbers of 10^8 or 10^9 cells



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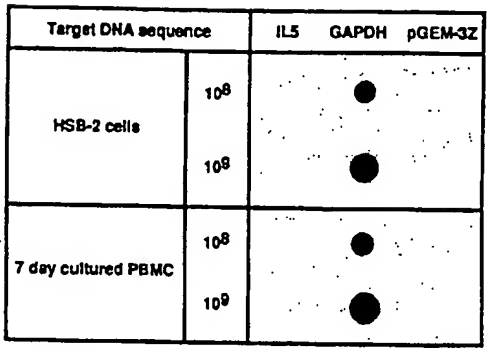

Fig. 1. Analysis of transcription by the conventional nuclear run on assay. Nuclei were extracted from either 10^8 or 10^9 HSB-2 cells stimulated with PMA and ionomycin for 4 h. Transcripts were ^{32}P-labelled by elongation in vitro, extracted and hybridised to the indicated IL-5 or GAPDH cDNA fragments that had been blotted onto the membrane. Hybridisation to pGEM-3Z without any cDNA insert was also assessed and was not detected.

were utilised (Fig. 1). The presence of readily detectable GAPDH transcription in this assay indicated that the nuclei were intact and that transcription was occurring.

3.2. Novel PCR based nuclear run on assay

A run on assay was developed with the following modifications: nuclei were split into two aliquots, one of which contained all four rNTPs and the other had no rNTPs; no radioactive UTP was used; at the end of the in vitro transcription reactions, products were detected by RT-PCR. All other aspects of the nuclear extraction and transcription reactions were identical to the conventional assay. HSB-2 cells were activated and after various times nuclei were harvested for the novel run on assay. No IL-5 transcripts were detected in unstimulated cells either in the presence or absence of transcription reactions (Fig. 2A, 0 h, + and − transcription reaction, respectively). When nuclei were harvested 2 h after stimulation, IL-5 RNA was detected in the sample with rNTPs, with little or no signal in the sample without rNTPs. In nuclei taken 4 h after stimulation, IL-5 signals were present in both samples. In cells that were cultured for 16 h after activation prior to harvesting the nuclei, little IL-5 RNA was detected in either sample (Fig. 2B). Total cellular IL-5 mRNA

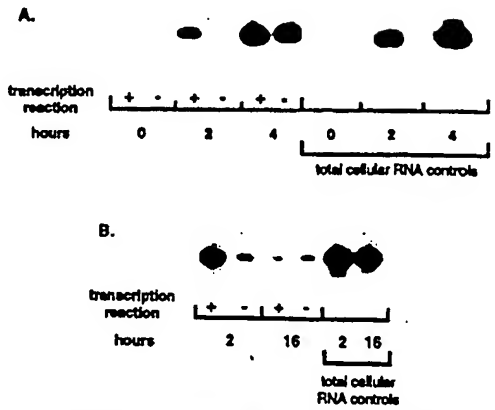

Fig. 2. Kinetics of IL-5 transcription. Nuclei were isolated from 10^4 HSB-2 cells stimulated with PMA and ionomycin for 0 to 4 h (*A*) and 2 and 16 h (*B*). Nuclei were incubated for 30 min with (+transcription reaction) or without (−transcription reaction) rNTPs. RNA was extracted, RT-PCR was performed and transcripts were detected by gel electrophoresis and Southern hybridisation with an internal ^{32}P-labelled probe. Total cellular RNA was analysed by RT-PCR to assess IL-5 mRNA abundance at the equivalent time points. Results are representative of two independent experiments.

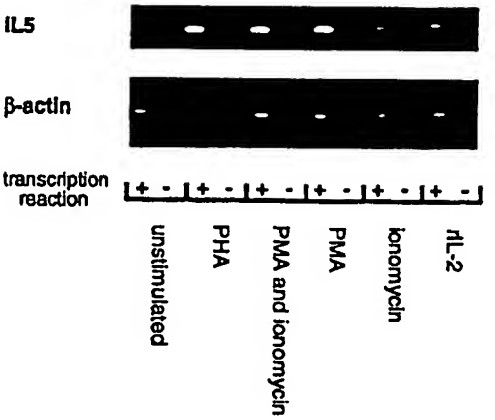

Fig. 3. Effect of different signalling pathways on the rate of IL-5 transcription. Nuclei were extracted from 10^4 7-day-cultured PBMC and exposed for 3 h to medium alone (lanes a), PHA (lanes b), PMA and ionomycin (lanes c), PMA (lanes d), ionomycin (lanes e) or recombinant IL-2 (lanes f). The amount of IL-5 transcription was assessed as described in Fig. 2, except the transcripts were identified by size fractionation without Southern hybridisation. The amount of β-actin transcription was used as an internal control to ensure the integrity of the nuclei. Results shown are representative of three independent experiments.

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Target DNA sequence		IL5 GAPDH pGEM-3Z
HSB-2 cells	10^8	
	10^9	
7 day cultured PBMC	10^8	
	10^9	

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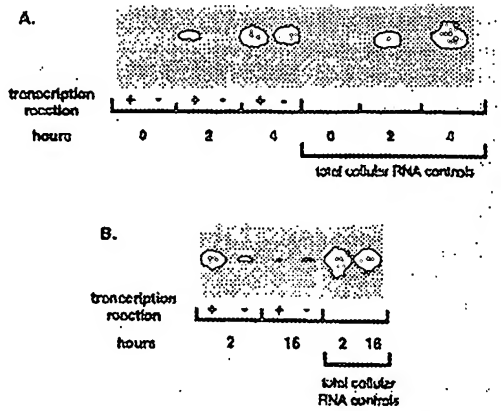

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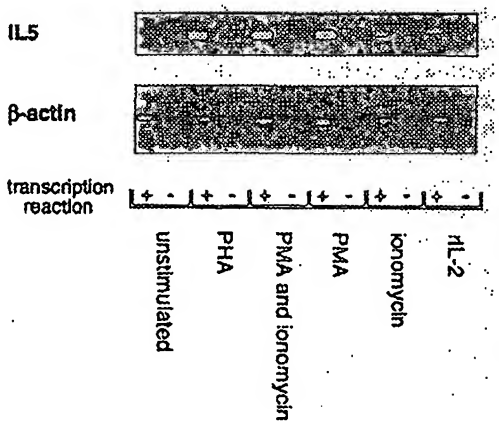

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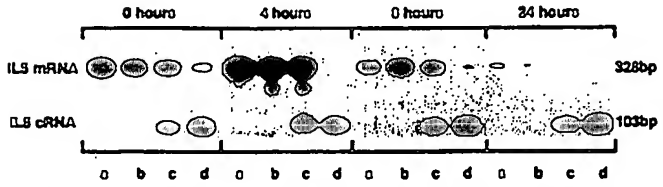

Fig. 4. RT-PCR of IL-5 mRNA and cRNA. HSB-2 cells (2×10^6 cells/ml) were cultured with PMA and ionomycin for 16 h. Cells were then harvested at 0, 4, 8 and 24 h after this time. Total cellular RNA was extracted and IL-5 mRNA expression analysed by quantitative RT-PCR. For each time point 4 reactions, consisting of 2×10^5 cell equivalents of cellular RNA and 10^5, 10^6, 10^7 or 10^8 competitor RNA molecules (lanes a, b, c and d respectively) were performed. The 328 bp IL-5 mRNA PCR fragment and the 103 bp cRNA fragment were identified by size fractionation and Southern hybridisation. The figure shown is representative of three experiments.

was also assessed. It was absent in the unstimulated sample, and readily detected in the stimulated cells at 2, 4 and 16 h after activation. Similar results were found with 7-day-cultured PBMC (data not shown).

3.3. Induction of IL-5 transcription via different signalling pathways

IL-5 mRNA expression is induced in human PBMC and 7-day-cultured PBMC in response to a range of stimuli (Rolfe et al., 1992). The effect of different agents on the rate of IL-5 gene transcription was investigated. 7-day-cultured PBMC were stimulated with medium alone, PHA, PMA and ionomycin, PMA alone, ionomycin alone or rIL-2 for 3 h. As shown in Fig. 3, there was no IL-5 transcription or IL-5 RNA in the nuclei in unstimulated 7-day-cultured PBMC. IL-5 gene transcription was induced in response to all stimuli. Stimulation of the cells with PHA, PMA and ionomycin and PMA alone gave the greatest rates of IL-5 transcription. β-Actin transcription was also analysed as a control. The amount of β-actin transcription in all of these samples, including the unstimulated sample, was similar, indicating they all had intact nuclei and equal constitutive transcription.

3.4. Analysis of the rate of IL-5 mRNA degradation

By 16 h after activation, ongoing IL-5 transcription could not be detected and little IL-5 RNA was present in the nuclei (Fig. 2). By contrast, IL-5 mRNA was detected in total cellular mRNA from 4 to 48 h after activation (Rolfe et al., 1992; Sewell et al., 1992). Therefore, 16 h after activation provided

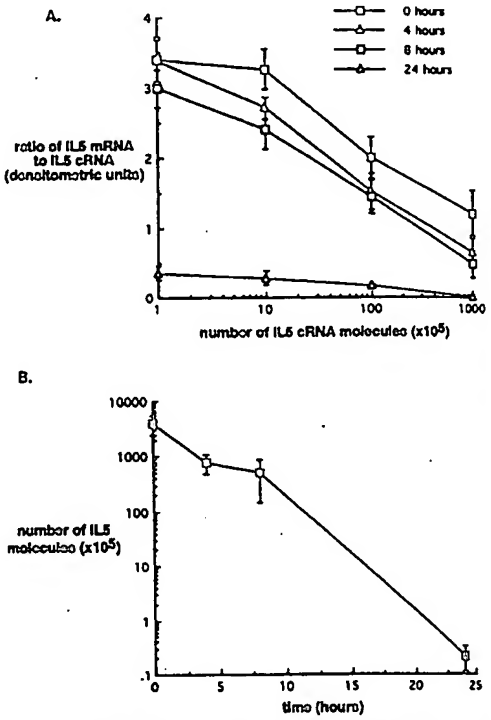

Fig. 5. Calculation of the number of IL-5 molecules in cellular RNA samples from stimulated HSB-2 cells. (*A*) The intensity of IL-5 mRNA and IL-5 competitor RNA signals was determined by densitometry. Their ratio was calculated and plotted against the number of competitor RNA molecules. The data are presented as mean ± SEM of three experiments, of which Fig. 4 is representative. (*B*) The number of IL-5 mRNA molecules in the cellular RNA samples was calculated from the point where the ratio equals 1 in the graph in part (*A*). For each time point the absolute number of IL-5 mRNA molecules was calculated and plotted against the time in hours.

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4. Discussion

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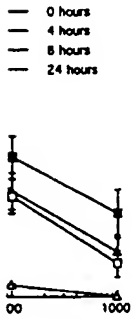

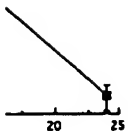

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an ideal starting time point for studies on the rate of IL-5 mRNA degradation. HSB-2 cells were activated with PMA and ionomycin for 16 h. Cells were harvested at 0 (equivalent to 16 h post-stimulation), 4, 8 and 24 h later and total cellular RNA was extracted.

2×10^5 cell equivalents of RNA for each time point were combined with titrated amounts of IL-5 cRNA, ranging from 10^5 to 10^8 molecules, and subjected to RT-PCR. IL-5 mRNA and cRNA were separated by gel electrophoresis and identified by Southern hybridisation as shown in Fig. 4. Densitometric analysis was performed to determine the ratio of IL-5 mRNA to IL-5 cRNA. The ratio was then plotted against the number of IL-5 cRNA molecules, as presented in Fig. 5A. The number of IL-5 mRNA molecules in the stimulated HSB-2 cells at the 0, 4, 8 and 24 h time points is shown in Fig. 5B. At the 4-h time point (equivalent to 20 h after stimulation), the number of IL-5 mRNA molecules in an activated HSB-2 cell ranged from 156 to 189 ($n = 10$). The half-life of IL-5 mRNA, that is the time taken for half the number of IL-5 molecules to decay, after PMA and ionomycin stimulation was 2.6 ± 0.3 h in HSB-2 cells and 4.0 ± 0.4 h in 7-day-cultured PBMC ($n = 6$).

4. Discussion

IL-5 transcription could not be detected with the conventional nuclear run on assay (Fig. 1). We therefore developed a novel assay based on the PCR. Because the run on products are not labelled, transcription that takes place in the isolated nuclei must be distinguished from RNA already present in the nuclei prior to isolation. This is achieved by including control samples, lacking rNTPs, for every transcription reaction. In such samples, shown as '− transcription reaction' in Fig. 2 and 3, IL-5 signals were not detected in nuclei from unstimulated cells, were weak or absent in cells stimulated for 2–3 h, were readily detected at 4 h, and were weak by 16 h (Fig. 2). In Figs. 2 and 3, the PCRs were terminated when products in the '+ transcription reaction' tracks were still accumulating exponentially, providing a more accurate distinction between the '+' and '−' tracks. The total cellular RNA signal was absent at 0 h, rose from 2 to 4 h and was readily detectable at 16 h. In activated cells, nearly all the IL-5 mRNA is in the cytoplasm (data not shown). However, it is very unlikely that the '− transcription reaction' tracks represent contamination of the nuclear samples with cytoplasmic RNA, because at 16 h total cellular RNA was abundant when the '− transcription reaction' signal was weak (Fig. 2).

In unstimulated T cells, no IL-5 RNA was present in the nucleus prior to isolation and no new transcription was detected (Figs. 2 and 3). This is consistent with the absence of IL-5 mRNA in RT-PCR on total cellular RNA of unstimulated T cells (Fig. 2; Rolfe et al., 1992; Sewell et al., 1996). In these cells, although there was no IL-5 transcription, β-actin transcription was readily detected (Fig. 3), consistent with findings on total cellular mRNA (Rolfe et al., 1992). At 2 h after activation, IL-5 transcripts were much more readily detected in the samples with transcription reactions than in those without (Fig. 2). This is consistent with the appearance of detectable cellular IL-5 mRNA from this time onwards. At later time points, the abundance of IL-5 transcripts was similar in the samples with and without transcription reactions (Fig. 2), suggesting that ongoing IL-5 transcription had ceased and that the assay was detecting transcripts formed in the nuclei prior to the nuclear isolation. We conclude that the amount of ongoing transcription in the cells at the time of nuclear harvest is represented by the difference between the samples with and without transcription reactions. IL-5 transcription is predominantly occurring within the first 4 h after activation.

The primers used in the present experiments give rise to a 328 bp PCR product from completely spliced cDNA and a 1.5 kb fragment from genomic DNA. In the PCR based nuclear run on assay, the size of the principal products was consistent with complete splicing, and larger PCR products were not detected (Figs. 2 and 3). It is commonly believed that the splicing machinery in isolated nuclei is inefficient, resulting in the presence of unspliced transcripts. For example, in a recent description of the use of RT-PCR as a measure of gene transcription, analysis was based on the detection of unspliced RNA (Elferink and Reiners, 1996).

There are two possibilities for the predominance of spliced products with the novel method. Firstly,

splicing may not be completely absent in isolated nuclei. Intact splicing machinery can be recovered from nuclei prepared with techniques similar to those employed in the present experiments (Eperon and Krainer, 1994). Secondly, in isolated nuclei, any transcription in the '+ transcription reaction' samples, even initiated at strand breaks, would provide fragments which could act as primers for preformed mature transcripts. It should be noted that in the '− transcription reaction' samples, if the PCR was continued for several more cycles, β-actin was always detected and IL-5 was detected in all cases except unstimulated cells. Therefore mature transcripts are present in the nucleus in low abundance, and are available to act as templates in the PCR. In either of these two possibilities, the PCR conditions, which were not designed to facilitate the production of the longer unspliced products, would favour amplification of completely spliced transcripts, even if they were present in low amounts.

The novel PCR based nuclear run on assay has certain advantages over the conventional assay. Most notably there is increased sensitivity, with IL-5 transcription detected in experiments with as few as 10^4 cells (Fig. 2). This contrasts with the absence of detectable IL-5 transcription in 10^9 cells using the conventional nuclear run on assay (Fig. 1). This method is potentially applicable to other genes expressed at low abundance, such as cytokine genes. The method is amenable to quantitation, by performing an analysis such as the quantitative RT-PCR described in this paper, on RNA extracted from samples at the conclusion of the nuclear run on assay.

The lack of constitutive transcription in unstimulated cells (Figs. 2 and 3) suggests that the absence of IL-5 mRNA in such cells is not due to rapid mRNA degradation. By contrast, the related cytokines IL-3 and IL-4, whose genes are in the same chromosomal region as IL-5 (van Leeuwen et al., 1989), are transcribed and rapidly degraded in unstimulated cells (Ryan et al., 1991; Dokter et al., 1993). As there is no evidence for basal IL-5 gene transcription, then all stimuli of IL-5 mRNA accumulation would be expected to induce IL-5 gene transcription, as was observed (Fig. 3). IL-5 cellular mRNA is relatively stable (Figs. 4 and 5), accounting for its persistence for 24–48 h after activation (Rolfe et al., 1992), even though it is only transcribed within the first 4 h after activation (Figs. 2 and 3) Murine IL-5 gene expression is regulated predominantly at the level of gene transcription (Naora and Young, 1994). The experiments reported in this paper, taken together, indicate that human IL-5 expression is also principally regulated in this way.

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